Journal of Physiology (1990), **424**, *pp.* 411–426
With 8 figures
Printed in Great Britain

EFFECTS OF METABOLIC BLOCKADE ON THE REGULATION OF INTRACELLULAR CALCIUM IN DISSOCIATED MOUSE SENSORY NEURONES

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(*Received 9 October 1989*)

SUMMARY

1. Impaired intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) regulation may underlie alterations in neuronal function during hypoxia or hypoglycaemia and may initiate cell damage. We have used the Ca^{2+}-sensitive fluorophore, Fura-2, to study the regulation of $[Ca^{2+}]_i$ in neurones isolated from mouse dorsal root ganglia. Mean resting $[Ca^{2+}]_i$ was 163 ± 11 nM (mean $\pm$ S.E.M., $n = 38$).

2. Depolarization by exposure to 20 or 30 mM-K^+ caused a rapid Co^{2+}- and Cd^{2+}-sensitive rise in $[Ca^{2+}]_i$, which subsequently declined with a time course usually fitted by the sum of two exponential functions.

3. Interference with mitochondrial function (by CN^- or FCPP) or with glycolysis (by glucose removal) all raised $[Ca^{2+}]_i$ by up to 220%. Addition of FCCP in the presence of CN^- further increased $[Ca^{2+}]_i$. The response to CN^- was still seen in the absence of extracellular Ca^{2+}, although it attenuated rapidly, indicating release from an intracellular store.

4. Either CN^- or glucose removal increased the rise in $[Ca^{2+}]_i$ induced by K^+ 2- to 3-fold and slowed recovery, suggesting interference with sequestration or extrusion of $[Ca^{2+}]_i$.

5. Resting $[Ca^{2+}]_i$ rose when external Na^+ was replaced by Li^+ or *N*-methyl-D-glucamine, demonstrating the presence of a Na^+–Ca^{2+} exchange process. However, Na^+ replacement had only a slight effect on the handling of a Ca^{2+} load.

6. We conclude that (i) Ca^{2+} is released into the cytoplasm from intracellular organelles when energy supplies are reduced; (ii) that the extrusion or sequestration of Ca^{2+} entering the cell during electrical activity is rapidly impaired by interference with mitochondrial metabolism; and (iii) Na^+–Ca^{2+} exchange makes only a small contribution to intracellular Ca^{2+} homeostasis.

7. $[Ca^{2+}]_i$ would thus be expected to rise *in vivo* during hypoxia or hypoglycaemia and may initiate alterations in neuronal function. However, if a rise in Ca^{2+} is an important cause of cell damage in cerebral hypoxaemia, the combination of

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excitation and hypoxia will lead to the largest increases in $[Ca^{2+}]_i$, while hypoxia alone appears to cause only a small increase in $[Ca^{2+}]_i$ in quiescent cells.

INTRODUCTION

The tight regulation of intracellular Ca^{2+} in all cells in essential to allow Ca^{2+} to act as a useful signal. This is especially true in neurones, where changes in $[Ca^{2+}]_i$ are central to many fundamental processes. It has been suggested that increases in $[Ca^{2+}]_i$ due to impairment of regulatory processes might underlie at least some of the changes in CNS function that arise during metabolic deprivation due to hypoxia or hypoglycaemia (Krnjević, 1975; Duchen, 1990; for reviews see Siesjö, 1981; Somjen, 1989) including the triggering of events that lead to cell death (Schanne, Kane, Young & Faber, 1979). In the present study we have attempted to identify ways in which $[Ca^{2+}]_i$ is regulated by neurones freshly dissociated from the dorsal root ganglia of the mouse. These cells have been used for a parallel electrophysiological study so that the measurements of $[Ca^{2+}]_i$ following various manipulations may therefore be related directly to the electrophysiological consequences of those manipulations (Duchen, 1990). We have studied the consequences of interference with cell metabolism and Na^+–Ca^{2+} exchange for $[Ca^{2+}]_i$, in order both to identify the roles of different Ca^{2+} uptake and extrusion mechanisms in normal $[Ca^{2+}]_i$ regulation and also to identify pathophysiological changes in $[Ca^{2+}]_i$ that might occur during the clinical states of hypoxia and hypoglycaemia.

A preliminary report of some of these data has been published (Biscoe, Duchen, Eisner, O'Neill & Valdeolmillos, 1988).

METHODS

Brief descriptions of the preparation have been published previously (Duchen & Somjen, 1988; Duchen & Pearce, 1989). Mice age 12–16 days were killed by decapitation, and the spinal column was removed into an ice-cold solution containing (mM): NaCl, 130; $NaHCO_3$, 26; KCl, 3·0; KH_2PO_4, 1·25; $CaCl_2$, 2·0; $MgCl_2$, 2·0; D-glucose, 10; pH 7·3. The solution was continuously bubbled with 95% O_2–5% CO_2. A ventral laminectomy exposed the dorsal root ganglia, which were carefully removed into a 2 ml aliquot of a similar solution containing 0·2% collagenase type II (Sigma), in which the ganglia were incubated at 35 °C for 30 min. They were then transferred to a similar solution which contained papain 0·035% (Cooper) and cysteine (175 μg/ml, Sigma) also at 35 °C. Ganglia were removed at any time after a 30 min incubation in papain, into a HEPES-based saline, similar to the above but with 156 mM-NaCl, no $NaHCO_3$, and 7·5 mM-HEPES, pH 7·4. Cells were then dispersed by trituration with sequentially finer tipped flame-polished Pasteur pipettes. The viability of cells prepared in this way has been demonstrated using whole-cell patch-clamp recording techniques (Duchen & Somjen, 1988; Duchen & Pearce, 1989; Duchen, 1989, 1990). Cells maintain resting potentials of -55 to -65 mV and express the range of voltage- and transmitter-gated membrane currents expected from other electrophysiological studies of cells in whole ganglia or grown in culture. Most of the recordings in this study were made from the larger cells present in the preparation (30–50 μm diameter) which would probably be classified as IA cells, but no differences were apparent in the responses of smaller cells.

Cells were loaded with Fura-2 (Molecular Probes) by incubation with the acetoxymethyl ester (10 μM) for 10 min at 35 °C, and were then kept on ice. Fluorescence measurements were made as previously described, using a spinning wheel (Eisner, Nichols, O'Neill, Smith & Valdeolmillos,

1989) to allow switching of excitation wavelengths between 340 and 380 nm. Fluorescence from single cells was measured at 500 nm, using a diaphragm to isolate the image of the cell from the background and from other cells. The ratio of fluorescence excited at 340 nm to that at 380 nm was used to obtain measurements directly related to $[Ca^{2+}]_i$ distinct from other causes of altered fluorescence , such as loss of the dye, as, in this instance, changes in fluorescence occur equally at both wavelengths (see Grynkiewicz, Poenie & Tsien, 1985). Bleaching of the signal, which may alter the measured ratio (Becker & Fay, 1987) was not significant over the time course of these experiments. The cells were continuously superfused and drugs were applied or solutions changed by switching between reservoirs. The small bath and the high flow rates used allowed reasonably rapid (seconds) exchange of solutions. Drugs used included sodium cyanide (CN^-, Sigma; made up to 2 mM and pH adjusted to 7·4 with the addition of HEPES), FCCP (1 μM), and caffeine (10 mM) (all from Sigma). In some experiments the solution were changed to high-K^+ or Na^+-free saline solutions. In the former case, KCl replaced NaCl, and in the latter, LiCl or *N*-methyl-D-glucamine (NMG) replaced NaCl.

In some experiments the pH-sensitive dye BCECF (Molecular Probes; Rink, Tsien & Pozzan, 1982) was used. Cells were again loaded with the dye by incubation with the acetoxymethyl ester. Fluorescence was excited in this case at 430 and 500 nm, and emitted light was measured at 530 nm. Again, the ratio of fluorescence excited at the two wavelengths was used to obtain measurements of pH_i.

Calibration of most of the fluorescence signals was achieved using an *in vitro* calibration procedure (see Biscoe, Duchen, Eisner, O'Neill & Valdeolmillos, 1989). Fura-2 (5 μM) as the free acid was added to saline solution containing Ca^{2+}–EGTA buffers giving minimum and saturating levels of Ca^{2+}, allowing determination of the K_D, and the minimum and maximum fluorescence ratios, required for the equation:

$$[Ca^{2+}]_i = K_D \beta(R - R_{min})/(R_{max} - R),$$

where β is the ratio of the maximum/minimum fluorescence excited at 380 nM. The value obtained for the K_D in our system was close to 100 nM. *In vivo* calibrations were achieved using the Ca^{2+} ionophore bromo-A23187 (2–10 μM; Calbiochem) to permeabilize cells to Ca^{2+}, followed by exposure to a range of Ca^{2+}–EGTA buffers, again giving different known Ca^{2+} concentrations. Calibration of BCECF fluorescence was achieved using nigericin (Sigma; 10 μM) to permeabilize the cells and exposing the cells to a range of saline solutions at different pH values (see Biscoe *et al.* 1989).

All experiments were performed at room temperature (18–22 °C).

RESULTS

Changes in $[Ca^{2+}]_i$ *in response to* K^+*-induced depolarization*

Measurements of resting $[Ca^{2+}]_i$ ranged from 40 to 200 nM with a mean of 163 nM ($\pm$11 nM, S.E.M., $n = 38$). Depolarization of cells by exposure to 20 or 30 mM-K^+ (Fig. 1*A*) raised $[Ca^{2+}]_i$ by 256$\pm$70 nM (mean$\pm$S.E.M., $n = 11$) above the resting level. The variability of this response suggests variability in cell resting potentials and Ca^{2+} current amplitudes. In some cells, the exposure was maintained, and the $[Ca^{2+}]_i$ level began to decline slowly, presumably reflecting a combination of inactivation of Na^+ and Ca^{2+} currents, and activation of K^+ conductances, balanced by Ca^{2+} removal and/or sequestration. The rate of recovery of Ca^{2+} on return to normal $[K^+]_o$ could usually be fitted by the sum of two exponentials (Fig. 1), although the relative contributions of the processes giving rise to each rate constant varied considerably between preparations. The rise in $[Ca^{2+}]_i$ following depolarization was almost completely blocked by superfusion with 100 μM-$CdCl_2$ or 2 mM-$CoCl_2$ (Fig. 1*B*), confirming that the Ca^{2+} enters through voltage-gated channels.

Effects of metabolic inhibitors on resting $[Ca^{2+}]_i$

Exposure of cells to 2 mM-CN^-, which blocks mitochondrial electron transport, to the mitochondrial uncoupling agent FCCP, or to glucose-free solutions, all increased resting $[Ca^{2+}]_i$. CN^- raised $[Ca^{2+}]_i$ by 79 ± 13 nM ($n = 18$) and glucose removal by

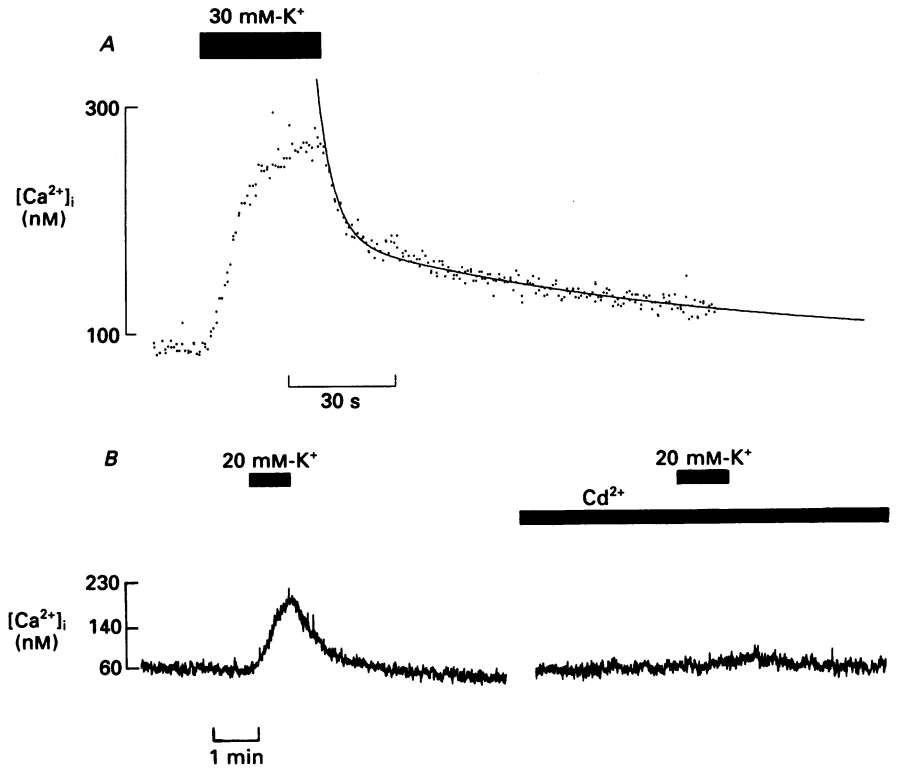

Fig. 1. *A*, exposure of a single dorsal root ganglion cell to a solution containing 30 mM-K^+ raised $[Ca^{2+}]_i$ from a resting level of about 100 nM to about 290 nM. On returning to the control superfusate, $[Ca^{2+}]_i$ fell gradually back to control values. The recovery was fitted well by the sum of two exponentials (continuous line) of the form:

$$y = 84{\cdot}3e^{-t/4{\cdot}62} + 93{\cdot}7e^{-t/106} + 98.$$

B, the K^+-induced rise in $[Ca^{2+}]_i$ could be completely blocked by superfusion with 100 μM-Cd^{2+} (or 2 mM-Co^{2+}), confirming that the rise in $[Ca^{2+}]_i$ is due to entry through voltage-gated channels.

124 ± 25 nM ($n = 6$). In Fig. 2*A*, the increase in $[Ca^{2+}]_i$ following exposure to CN^- is shown in comparison to a response to K^+-induced depolarization. The responses to CN^- were never as large as those induced by depolarization, and the rate of onset was typically much slower, although this rate varied considerably between preparations (see, for example, Fig. 3). When exposure to CN^- was prolonged, as shown in this figure, $[Ca^{2+}]_i$ initially rose rapidly, and then continued to rise more slowly until the CN^- was removed. The rates of recovery from CN^- and from depolarization were similar. Exposure to glucose-free solutions similarly raised $[Ca^{2+}]_i$ (Fig. 2*B*), with an

initial rapid rise to a new level which either remained constant (Fig. 2*B*) or continued to rise slowly. In most cases the response to any of these manoeuvres was fully reversible.

The rise in $[Ca^{2+}]_i$ seen in response to CN^- was not maximal, and could be further increased by the addition of FCCP, as illustrated in Fig. 3. Exposure to CN^- raised $[Ca^{2+}]_i$ which then stayed relatively stable. Application of 1 μM-FCCP in the presence of CN^- caused a further rise in $[Ca^{2+}]_i$. Removal of both agents was eventually followed by full recovery to control values.

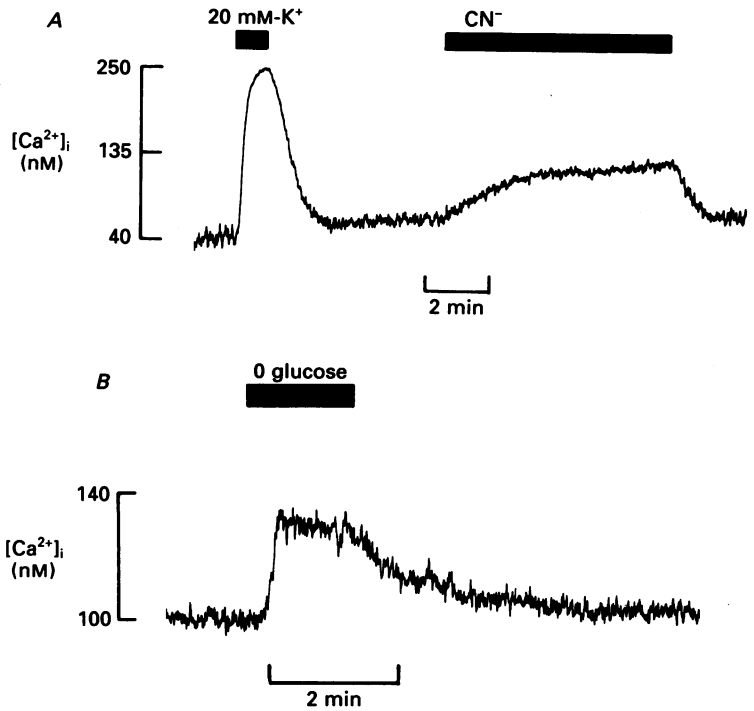

Fig. 2. *A*, responses to 20 mM-K^+ and to 2 mM-CN^-. Resting values of $[Ca^{2+}]_i$ of about 40 nM rose to 250 nM in response to depolarization and recovered rapidly. Subsequent exposure to CN^- raised $[Ca^{2+}]_i$ to about 120 nM within 2 min. The new elevated level continued to rise slowly until CN^- was removed. *B*, in this cell, exposure to a glucose-free solution caused a rapid and reversible rise of $[Ca^{2+}]_i$.

An increase in resting $[Ca^{2+}]_i$ may result from: (i) increased Ca^{2+} entry into the cell; (ii) impaired extrusion of Ca^{2+} from the cytosol due to impaired activity of ATP-dependent Ca^{2+} pumps in the cell membrane in the face of a constant influx either through voltage-gated channels (if cells are spontaneously active) or through some other leakage pathway; (iii) impaired sequestration of Ca^{2+} into intracellular organelles, again in the face of a constant influx of Ca^{2+}; (iv) a fall in [ATP] may lead to impaired activity of the Na^+–K^+-ATPase, leading to Na^+ accumulation and reversal of Na^+–Ca^{2+} exchange; or (v) Ca^{2+} may be released from an intracellular pool.

Removal of extracellular Na^+ increased $[Ca^{2+}]_i$, demonstrating the presence of a

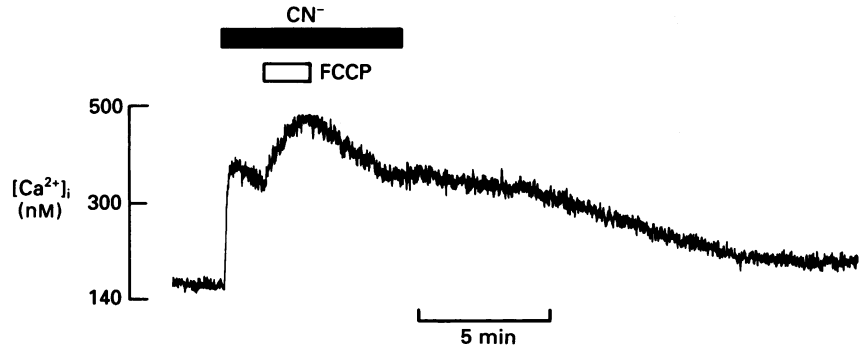

Fig. 3. After CN^- raised $[Ca^{2+}]_i$, a small further, reversible rise was seen after application of 1 μM-FCCP.

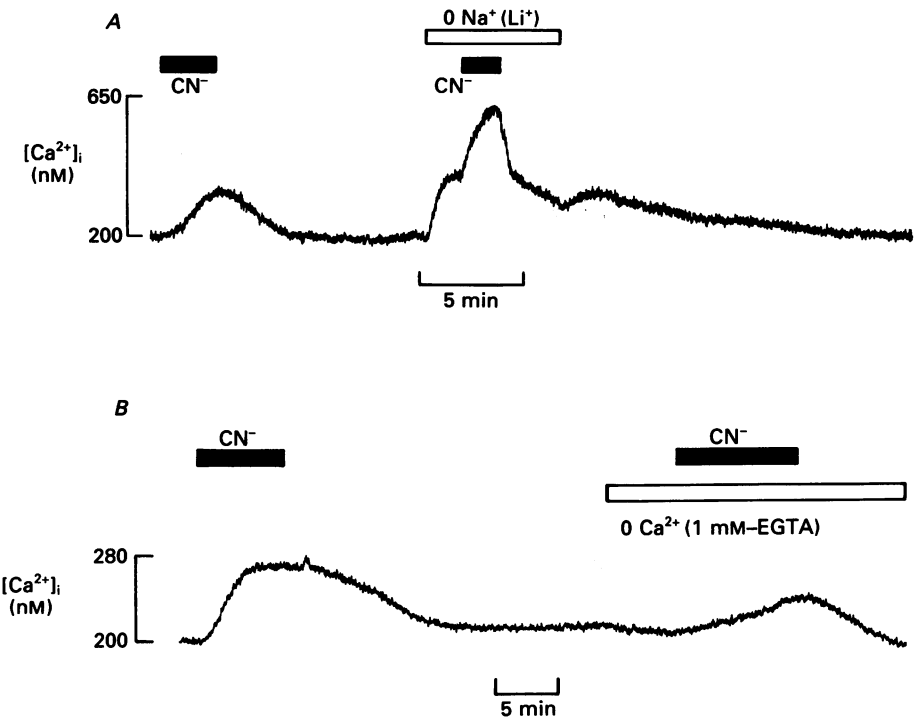

Fig. 4. *A*, the CN^--induced rise in $[Ca^{2+}]_i$ was independent of extracellular Na^+. Exposure to CN^- raised Ca^{2+}. Replacement of Na^+ in the superfusate with Li^+ also raised $[Ca^{2+}]_i$, demonstrating the presence of Na^+–Ca^{2+} exchange. Exposure to CN^- in the presence of Li^+ caused a further reversible rise in $[Ca^{2+}]_i$. *B*, the rise in $[Ca^{2+}]_i$ in response to CN^- was still seen after removal of extracellular Ca^{2+}, showing that CN^- increases the efflux of Ca^{2+} from an intracellular pool. Resting $[Ca^{2+}]_i$ fell slightly on removal of extracellular Ca^{2+}.

Na^+–Ca^{2+} exchange mechanism in these cells. The experiment illustrated in Fig. 4*A* shows that the effect of CN^- was independent of the activity of Na^+–Ca^{2+} exchange. Exposure of a cell to CN^- raised Ca^{2+} as before. Replacement of Na^+ with Li^+, which is not a substrate for the Na^+–Ca^{2+} exchange, raised $[Ca^{2+}]_i$. Further application of CN^- in the presence of Li^+ again raised Ca^{2+} reversibly. On returning to extracellular Na^+ in this experiment there was a small rise in $[Ca^{2+}]_i$ for which we cannot account, following which $[Ca^{2+}]_i$ recovered to its initial value. Thus, the response to CN^- was independent of extracellular Na^+, and cannot reflect intracellular Na^+ accumulation due to failure of Na^+–K^+ exchange.

The experiment illustrated in Fig. 4*B* shows that at least some of the Ca^{2+} contributing to the response to CN^- results from release from an intracellular pool, as a response could still be seen after removal of extracellular Ca^{2+}. When the superfusate was switched to a solution that contained submicromolar Ca^{2+} (no added Ca^{2+}, 1 mM-EGTA), $[Ca^{2+}]_i$ fell slightly, but if CN^- was applied soon after removal of extracellular Ca^{2+}, the response could still be seen. Subsequent applications of CN^- failed to evoke any response, suggesting that organelle, cytoplasmic and extracellular Ca^{2+} exchange rapidly, depleting the organelle store under these conditions.

There are several ready explanations for these observations: (i) inhibition of oxidative phosphorylation may cause an intracellular acidosis, leading to displacement of Ca^{2+} from binding sites by H^+ ions; (ii) Ca^{2+} is released from a microsomal storage site due to a fall in ATP or a rise in ADP, leading to failure to maintain the intracellular concentration gradient; or (iii) Ca^{2+} is released from mitochondria, due to a fall in the mitochondrial membrane potential, as mitochondrial Ca^{2+} uptake is potential dependent (Rottenberg & Scarpa, 1974).

We have excluded the first of these explanations. Using the pH-sensitive dye BCECF, to measure intracellular pH (pH_i), we have found that application of CN^- or removal of glucose fail to change intracellular pH detectably, although other manoeuvres, such as application of weak acids or bases, application of ammonium chloride, or bubbling the superfusate with 5% CO_2, all produced predictable changes in pH_i (not shown). The second argument is less readily dismissed. However, we have no evidence for the existence of a significant releasable microsomal fraction in this preparation. Application of caffeine (10 mM), which releases Ca^{2+} from sarcoplasmic or endoplasmic reticular stores (Weber & Herz, 1968; Kuba, 1980), failed to produce any detectable change in $[Ca^{2+}]_i$, even immediately after prolonged K^+-induced depolarization in an attempt to load intracellular stores (cf. Neering & McBurney, 1984).

Mechanisms for removal of a Ca^{2+} load

The rise in $[Ca^{2+}]_i$ resulting from K^+-induced depolarization was used to investigate the mechanisms available for Ca^{2+} removal or sequestration. Figure 5 shows the effect of CN^- on cellular handling of such an imposed Ca^{2+} load. Exposure to 20 mM-K^+ raised $[Ca^{2+}]_i$ reversibly. The relatively rapid rate of recovery was adequately fitted by a single exponential function as shown in the semilogarithmic plot shown in Fig. 5*B*. Resting $[Ca^{2+}]_i$ rose slightly when CN^- was applied, but the rise in $[Ca^{2+}]_i$ seen in response to the K^+ was now much greater than the control, and the rate of recovery was much slowed (Fig. 5*B*). On removal of the CN^-, the rate of recovery

quickly increased towards control values. The response to a further depolarization was slightly larger than the control, and the rate of recovery was almost back to control levels.

A similar protocol was used to examine the effect of glucose removal on Ca^{2+}

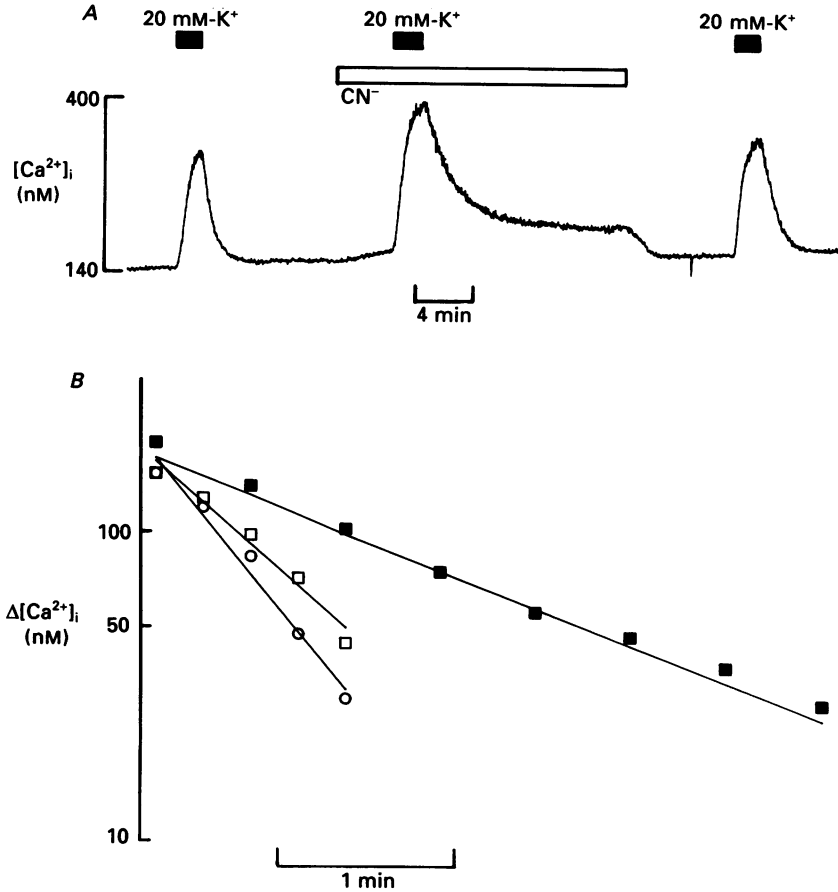

Fig. 5. Effect of CN^- on the handling of a Ca^{2+} load. *A*, depolarization with 20 mM-K^+ raised $[Ca^{2+}]_i$ reversibly. CN^- raised resting $[Ca^{2+}]_i$ slightly but the response to depolarization was increased. $[Ca^{2+}]_i$ recovered to a new level until CN^- was removed, but the rate of recovery was much slowed as shown in *B*. The recovery of $[Ca^{2+}]_i$ from depolarization could be fitted by a single exponential function. The control (○) was fitted by an equation of the form:

$$y = 162e^{-t/41.9}.$$

In the presence of CN^- (■), the equation became:

$$y = 199e^{-t/301},$$

and recovery (□) was:

$$y = 161e^{-t/57.9}.$$

handling, as shown in Fig. 6. The response in the exemplar cell illustrated was more dramatic but in principle similar to that seen with CN^-. The rise in $[Ca^{2+}]_i$ in response to depolarization was much increased, and again the rate of recovery, shown again

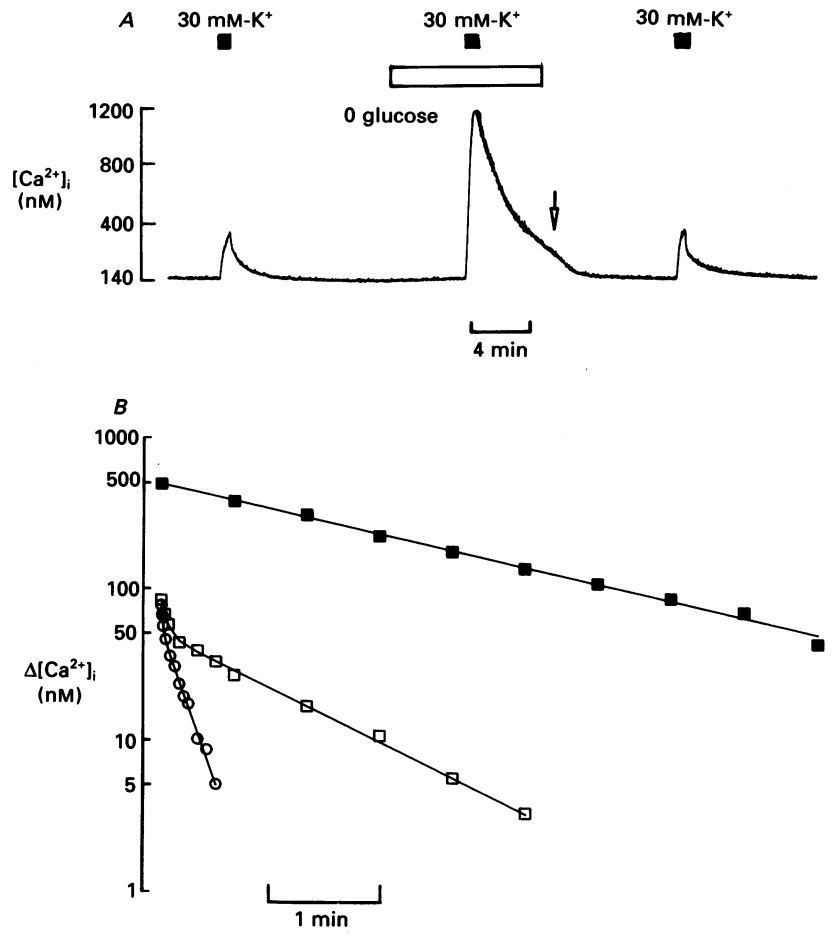

Fig. 6. The effect of glucose withdrawal on the response to depolarization. *A*, the original data are shown. Depolarization with 30 mM-K^+ raised $[Ca^{2+}]_i$. On removal of glucose, resting $[Ca^{2+}]_i$ rose slightly, but the response to depolarization was much increased. Recovery of $[Ca^{2+}]_i$ was considerably slowed until glucose was reintroduced to the superfusate. The response to a further depolarization was similar to the initial control. Recovery from the Ca^{2+} load was fitted by the sum of two expontential functions, as shown in *B*. In the control (○) the equation had the form:

$$y = 24{\cdot}6e^{-t/3{\cdot}85} + 52{\cdot}7e^{-t/51{\cdot}2} + 96{\cdot}6.$$

In the absence of glucose (■), the recovery was adequately fitted by a single exponential:

$$y = 491e^{-t/157} + 120.$$

Recovery (□) restored the early component and the initial level, but the second component remained slowed in this experiment, as the fit was:

$$y = 34{\cdot}4e^{-t/3{\cdot}36} + 49{\cdot}2e^{-t/73{\cdot}5} + 86{\cdot}4.$$

on a semilogarithmic scale, was significantly slowed compared to the control. The recovery from the initial control response in this instance was well fitted by the sum of two exponentials (see legend). In the absence of glucose, recovery showed only the one slow component which was also slower than the control. On returning

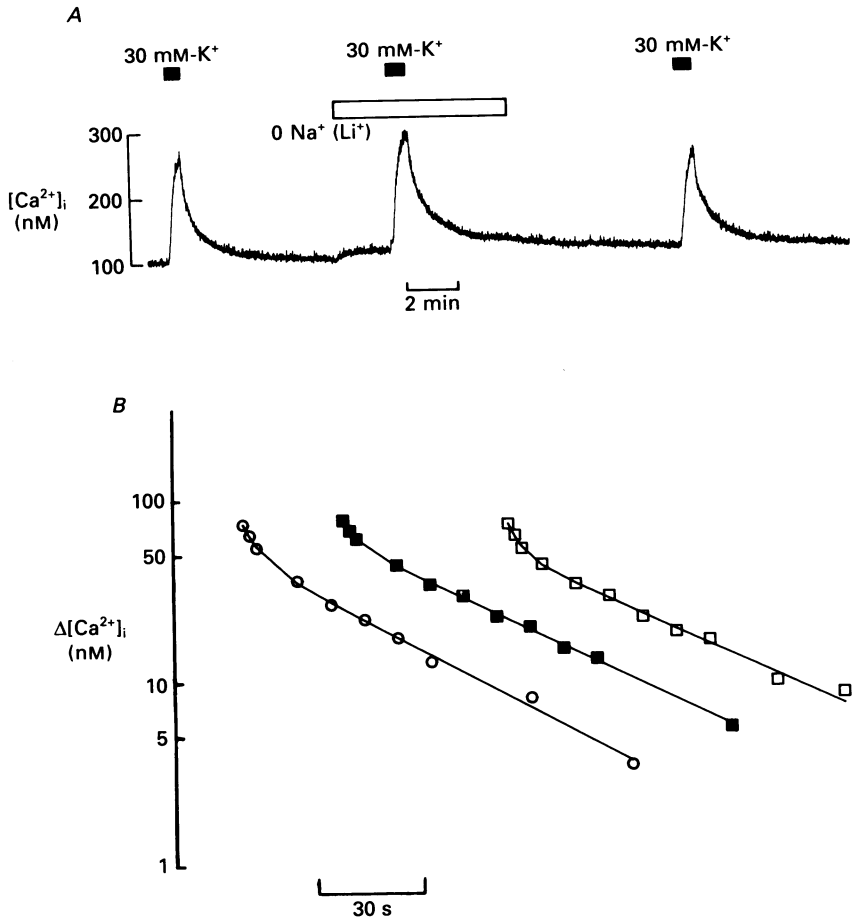

Fig. 7. Removal of extracellular Na^+ had no significant effects on the removal of a $[Ca^{2+}]_i$ load. The same protocol was used as in Figs 5 and 6. Recovery from K^+-induced depolarization was fitted by the sum of two exponential functions shown in *B* (○):

$$y = 27{\cdot}5e^{-t/4{\cdot}88} + 52{\cdot}9e^{-t/44} + 83{\cdot}1.$$

On replacement of Na^+ with Li^+, Ca^{2+} rose slightly. Depolarization again raised $[Ca^{2+}]_i$, but the rates of recovery were not significantly different from control (■), displayed in time to allow the curves to distinguished:

$$y = 19{\cdot}9e^{-t/3{\cdot}84} + 63{\cdot}4e^{-t/48{\cdot}8} + 101.$$

$[Ca^{2+}]_i$ remained slightly elevated until extracellular Li^+ was replaced with Na^+, and a further test with 30 mM-K^+ gave a response similar to the control fitted by:

$$y = 24{\cdot}6e^{-t/3{\cdot}85} + 52{\cdot}7e^{-t/51{\cdot}2} + 96{\cdot}6.$$

extracellular glucose, the recovery was again fitted by two exponentials, but the second, slow component was still slower than the initial control.

Figure 7 shows that records obtained when a similar sequence of manoeuvres was used to examine the role of Na^+–Ca^{2+} exchange in the removal of a Ca^{2+} load. Replacement of Na^+ with Li^+ (which will pass through Na^+ channels, and therefore does not prevent the generation of action potentials) caused a small rise in resting

$[Ca^{2+}]_i$. However, removal of Na^+ had relatively little effect on the handling of a Ca^{2+} load, barely changing the rise in Ca^{2+} produced by high K^+, and only slightly slowing the recovery, as shown on the semilogarithmic plots in Fig. 7*B*.

Figure 8 summarizes the changes in $[Ca^{2+}]_i$ seen in response to the various manoeuvres described.

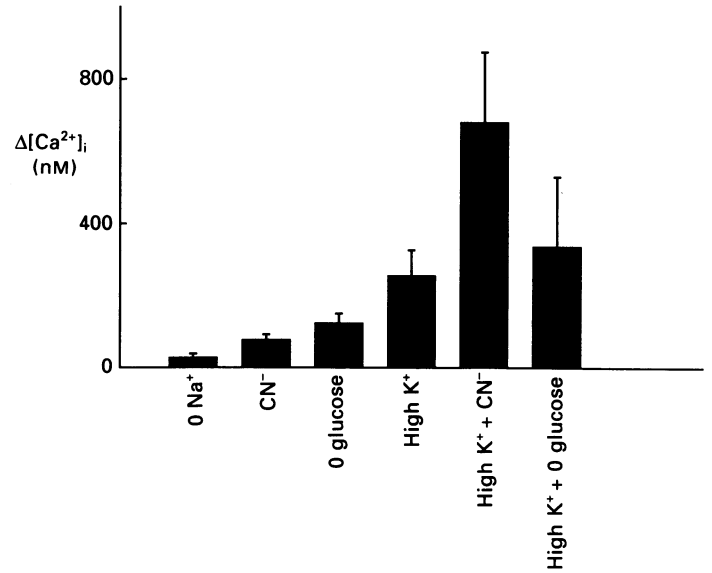

Fig. 8. This bar chart summarizes our findings, showing the extent of the elevation of $[Ca^{2+}]_i$ seen in response to the various manoeuvres described. The error bars show the S.E.M. The experimental numbers were: 0 Na^+, $n = 9$; CN^-, $n = 18$; 0 glucose, $n = 6$; high K^+, $n = 11$; high $K^+ + CN^-$, $n = 7$; high $K^+ + 0$ glucose, $n = 4$.

DISCUSSION

Our data show (i) that Ca^{2+} is released from an intracellular compartment in response to interference with mitochondrial or glycolytic function; (ii) that Na^+–Ca^{2+} exchange is relatively insignificant in intracellular Ca^{2+} homeostasis, and (iii) that changes in $[Ca^{2+}]_i$ in the clinical states of hypoxia or hypoglycaemia and the consequences for neuronal function may depend critically on the levels of Ca^{2+} loading of the cells. This will vary according to the electrical activity of the cells, on the membrane potential, or on the action of transmitters which promote Ca^{2+} influx.

Our results might best be explained in terms of altered mitochondrial Ca^{2+} uptake. The role of mitochondrial Ca^{2+} uptake in normal cellular Ca^{2+} homeostasis has been a contentious issue (Carafoli & Lehninger, 1971; Nicholls & Åkerman, 1981; Fiskum & Lehninger, 1982; McGraw, Nachshen & Blaustein, 1982; Nachshen, 1985; Carafoli, 1987). Our data show that there is a pool of Ca^{2+} within the cell that is released by metabolic inhibitors, possibly from the mitochondria. It remains possible that the source of Ca^{2+} is endoplasmic reticulum (ER), but the absence of a demonstrable caffeine-releasable pool in this preparation (see below) seems to make this less likely

unless a caffeine-insensitive endoplasmic reticular pool is postulated. Calcium accumulation in the ER is dependent on the activity of a Ca^{2+}–calmodulin-dependent ATPase. It is most unlikely that intracellular ATP levels fall significantly in the time scale of these experiments, certainly not to levels where the ATPase ceases to function, as the enzyme has a K_D for ATP in the micromolar range (Gill, Sheau-Huei & Whitlow, 1984; Gill & Chueh, 1985). Indeed, it seems likely that it is the sustained activity of the ATPases in the cell membrane or the ER (or both) that lower $[Ca^{2+}]_i$ following a Ca^{2+} load during maintained exposure to CN^- or glucose removal (see Figs 5 and 6).

Calcium enters mitochondria in response to an electrochemical gradient established by the electron transport chain (Rottenberg & Scarpa, 1974), so that mitochondrial respiration is a primary requisite for mitochondrial Ca^{2+} accumulation. It has been suggested that there is a continual cycle of Ca^{2+} between uptake and release by mitochondria (Crompton, Moser, Ludi & Carafoli, 1978; Carafoli, 1979; Crompton, 1985) so that impaired uptake will inevitably lead to increased net release from the mitochondrial pool, and will vary according to the rate of the cycle. Impaired uptake could also account for the increase in the K^+-induced Ca^{2+} load seen in the presence of CN^- or the absence of glucose (Fig. 6). While CN^- reduces the mitochondrial membrane potential, reversal of the ATPase may maintain a potential which is further depolarized by the proton ionophore FCCP, removing further the ability of mitochondria to maintain a Ca^{2+} store against the concentration gradient established between the cytoplasm and mitochondrial matrix (Brinley, Tiffert & Scarpa, 1978; Heinonen, Åkerman & Kaila, 1984).

According to this scheme, then, a fall in ATP is not a major prerequisite for Ca^{2+} release. This is consistent with the following: (i) in whole-cell patch-clamp recordings from these cells, the electrophysiological responses to CN^- or removal of glucose (increased Ca^{2+}-dependent K^+ conductance) is barely affected by the addition of 5 mM-MgATP or an ATP-regenerating system to the pipette-filling solution (Duchen, 1989); (ii) hypoxia leads to failure of synaptic transmission in hippocampal slices before a significant fall in ATP is detectable using NMR spectroscopy (Cox & Bachelard, 1982; Cox, Morris, Feeney & Bachelard, 1983); and (iii) exposure of synaptosomes to CN^-, FCCP or glucose removal depolarizes mitochondria well before the ATP/ADP ratio changes substantially (Kauppinen & Nicholls, 1986). Some other studies, however, do suggest that early effects of hypoxia may be associated with a fall in $[ATP]_i$ (Lipton & Wittingham, 1982).

The rise in $[Ca^{2+}]_i$ during these various metabolic insults is not due to depolarization of the cell membrane and activation of voltage-gated Ca^{2+} channels. Firstly, a rise in resting Ca^{2+} is still seen in the absence of extracellular Ca^{2+}. Secondly, in parallel electrophysiological studies, Duchen & Somjen (1988) and Duchen (1990) found that these cells are not depolarized, but hyperpolarize in response to all these agents, and that the hyperpolarization appears to be due to activation of a Ca^{2+}-dependent K^+ conductance. This suggests that the electrophysiological consequences of the metabolic insult reflects the change in Ca^{2+} homeostasis, and does not cause it.

The apparent absence of a caffeine-releasable intracellular Ca^{2+} store was

somewhat surprising. Neering & McBurney (1984) have demonstrated such a pool in rat dorsal root ganglia in culture, and showed that it was most readily demonstrable after Ca^{2+} loading by depolarizing the cells. In the freshly dissociated cells, even after raising $[Ca^{2+}]_i$ by potassium-induced depolarization we were unable to detect any caffeine-releasable Ca^{2+}, and must therefore conclude that there is some difference in the status of these intracellular organelles in cultured and in freshly dissociated cells.

The limited contribution of the Na^+–Ca^{2+} exchange in removing a Ca^{2+} load is somewhat at odds with the findings of Nachshen (1985) in synaptosomes and contrasts with findings in cardiac muscle (Reuter & Seitz, 1968), and in retinal rods (Cervetto, Lagnado, Perry, Robinson & McNaughton, 1989), where this mechanism is most important in intracellular Ca^{2+} homeostasis. This suggests that the relative importance of Na^+–Ca^{2+} exchange varies considerably between tissues, and may even differ between the neuronal somata and nerve terminals.

During hypoxia or hypoglycaemia, profound changes in CNS function follow rapidly on altered cell metabolism. Electrophysiological studies suggest that many neurones hyperpolarize in response to hypoxia or to mitochondrial poisons (Godfraind, Kawamura, Krnjević & Pumain, 1971; Krnjević, 1975; Hansen, Hounsgaard & Jahnsen, 1982; Fujiiwara, Higashi, Shimoji & Yoshimura, 1987; Krnjević & Leblond, 1987; Duchen & Somjen, 1988; Duchen, 1990). Studies of single-cell physiology with patch-clamp pipettes, which allow the introduction of ATP into the cell (Duchen, 1990), and of whole animals or brain slices with NMR (Cox & Bachelard, 1982), suggest that the early electrophysiological events precede changes in ATP availability, although this is not a view that is shared by all (Lipton & Whittingham, 1982). It seems increasingly likely that the hyperpolarization, due clearly to an increased K^+ conductance in all documented cell types, reflects activation of Ca^{2+}-dependent K^+ channels by the rising $[Ca^{2+}]_i$, although it remains possible that ATP-dependent K^+ channels (Noma, 1983) contribute in some cells.

It has been widely suggested that a rise in $[Ca^{2+}]_i$ underlies hypoxic cell death (Schanne *et al.* 1979; Deshpande, Siesjö & Wieloch, 1987; for review, see Siesjö, 1981). The rise in resting $[Ca^{2+}]_i$ demonstrated here seems unlikely to be sufficient alone to cause significant damage as it is very small in relation to the rise in $[Ca^{2+}]_i$ routinely provoked by depolarization. Most cells in the CNS are highly electrically active, and may experience higher levels of $[Ca^{2+}]_i$ during normal physiological activity than the small rise in $[Ca^{2+}]_i$ seen here in response to metabolic deprivation. However, it is also clear from this study that the combination of Ca^{2+} loading and metabolic insult may greatly amplify the elevation of $[Ca^{2+}]_i$, leading to levels which are more likely to initiate cell damage. This combination may occur in intact tissues through a variety of mechanisms, according to the following scheme (see also Somjen, 1989): (i) an increase in $[Ca^{2+}]_i$ activates Ca^{2+}-dependent K^+ channels. The increased K^+ conductance hyperpolarizes cells and shunts the membrane, keeping cells electrically quiescent. (ii) The rise in $[Ca^{2+}]_i$ may also increase the release of neurotransmitters (Van Harreveld, 1959; Katz, 1969; Alnaes & Rahamimoff, 1975; Benveniste, Drejer, Schousboe & Diemer, 1984). Released excitatory amino acids depolarize neurones and initiate a positive feedback system which will lead to further

depolarization, increased extracellular K^+ (Hansen, 1985) and increased Ca^{2+} loading, especially if NMDA receptors are activated (MacDermott, Mayer, Westbrook, Smith & Barker, 1986). (iii) Impaired glial function, both in terms of uptake of excitatory amino acids and K^+ regulation, may contribute to the overall picture. (iv) Eventually, of course, ATP levels will fall, both because mitochondrial respiration fails, but also because ATP utilization is increased as the mitochondrial proton pump consumes ATP to maintain the mitochondrial membrane potential. The fall in [ATP] will clearly lead to the final run-down of all energy-dependent processes, leading to cell death.

We gratefully acknowledge support from the Wellcome Trust, the Royal Society, and the British Heart Foundation. We also thank J. Dempster for the use of software for data fitting.

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